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13. ABSTRACT ***(Maximum 200 Words)***

Hoxb-13 is expressed in the spinal cord, hindgut, and urogenital sinus during embryogenesis. Accumulation of *Hoxb-13* mRNA is not diminished in prostate glands following castration indicating that it may be androgen independent. We have used a reporter gene approach in transgenic mice to search for cis-acting sequences that direct expression of *Hoxb-13* in the prostate. Elements that direct a pattern of reporter gene expression that closely parallels the endogenous gene during embryogenesis were identified within 8 kb upstream of the coding region. However, these elements failed to confer reporter gene expression in the prostate. Addition of further upstream and downstream sequences that together totaled nearly 20 kb in subsequent reporter constructs resulted in refinement of the embryonic pattern of *Hoxb-13* expression, but did not result in reporter gene activity in the prostate gland. Analyses of the *Hoxb-13* transcription unit revealed the nature of two sense transcripts that differ by virtue of differential polyadenlyation. An evolutionarily conserved antisense *Hoxb-13* transcript was also discovered. These data demonstrate that expression of *Hoxb-13* in the prostate gland is dictated by control elements that act at a distance, and raise the possibility of a complex mode of regulation involving an antisense mRNA.

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INTRODUCTION:

Hox genes encode the transcriptional regulatory proteins that are largely responsible for establishing the body plan of all metazoan organisms. A subset of Hox genes continues to be expressed during the period of organogenesis and into adulthood. *Hoxb-13* is a member of the Hox gene family that is expressed in the spinal cord, hindgut, and urogenital sinus during embryogenesis. We have characterized its expression in adult mouse tissues and have found that it is expressed in only two sites: the prostate gland and the distal colon. Surprisingly, accumulation of *Hoxb-13* mRNA is not diminished in prostate glands following castration indicating that its expression is androgen independent. In support of this suggestion, we have also demonstrated that the human *Hoxb-13* gene is expressed in androgen-independent PC-3 cells, as well as androgen starved LNCaP cells. Stimulation of LNCaP cells with androgen does not alter the expression of *Hoxb-13*. In our application, we proposed to characterize the molecular basis of prostate specificity and androgen independence of the *Hoxb-13* gene with a view towards developing new treatments for advanced prostate cancer. These elements would provide a unique reagent that could be incorporated into gene therapy strategies to treat advanced prostate cancer in patients that have undergone androgen deprivation therapy. Currently available prostate-specific regulatory elements are all androgen dependent and would be unlikely to function well in these patients.

BODY:

Task 1. To identify a region of the human *Hoxb-13* locus that is capable of directing expression of a reporter gene to prostatic epithelial cells in an androgen independent manner.

Restriction mapping of hoxb-13 loci: The first experimental objective relevant to this task was to develop a physical map of the human *Hoxb-13* genomic locus. To this end, two pairs of PCR primers were designed based on the sequence of *Hoxb-13* available in Genbank accession number HSU 57052. Conditions for amplification of 192 or 175 base pair products from human genomic DNA were then optimized. Following optimization, one pair of primers was sent to Genome Systems, Inc. to be used for screening a human PAC library for clones containing *Hoxb-13* sequences. This screen succeeded in identifying a single PAC clone containing an insert estimated to be >100 kb in size.

Subcloning of human and mouse genomic fragments: In preparation for construction of reporter genes carrying putative cis-regulatory regions of *hoxb-13* loci, we have subcloned multiple fragments containing both upstream and downstream regions of *Hoxb-13.* Although several of the fragments were readily cloned into conventional plasmid vectors, others proved to be refractory to cloning using standard technologies (see below).

Identification of cis-acting regulatory elements of the human Hoxb-13 gene that direct expression in mouse embryos: As outlined in our proposal, our initial efforts to locate prostate transcriptional control elements of *Hoxb-13* focused on sequences upstream of the coding region. The initial construct contained 8 kb of DNA extending from the translation start site to an upstream EcoRV restriction site. This construct was injected into FVB embryos, and 6 transgenic lines carrying the transgene were established. Analyses of lacZ reporter gene activity in transgenic embryos revealed strong reporter gene activity in a pattern that recapitulated the major aspects of the expected pattern for *hoxb-13* in 9.5-12.5 days post coitum (dpc) embryos (Figure 1 and data not shown). Strong ß-galactosidase (ß-gal) activity was observed in 9.5 dpc embryos in posterior somites, unsegmented mesoderm, and the developing spinal cord, in concordance with the pattern predicted by in situ hybridization studies. This pattern continued in 10.5 dpc embryos, however, ectopic expression was also observed in the myotome portion of somites anterior to the normal domain of *hoxb-13* expression. This observation indicates that the construct may lack a negative regulatory element that would preclude expression in the myotome. Although the intensity of staining varied among the three transgenic lines, the pattern was remarkably consistent. Deletion of the distal 4 kb from this transgene resulted in a construct that was not expressed in the expected *hoxb-13* pattern, and was instead active in a few cells in a pattern that varied from embryos to embryo (data not shown).

At 12.5 dpc, expression of the endogenous *hoxb-13* gene is observed in the developing urogenital sinus. Surprisingly, analyses of ß-gal activity in transgenic embryos carrying the 8 kb upstream construct failed to show reporter gene activity in the urogenital sinus region in both whole mount preparations and in histological sections. Analyses of ß-gal activity in neonatal and adult prostate glands also failed to show

measurable reporter gene activity. Consistent with these observations, Northern blot analyses of prostate RNA extracted from adult transgenic mice demonstrated that the reporter gene mRNA was not detectable (data not shown).

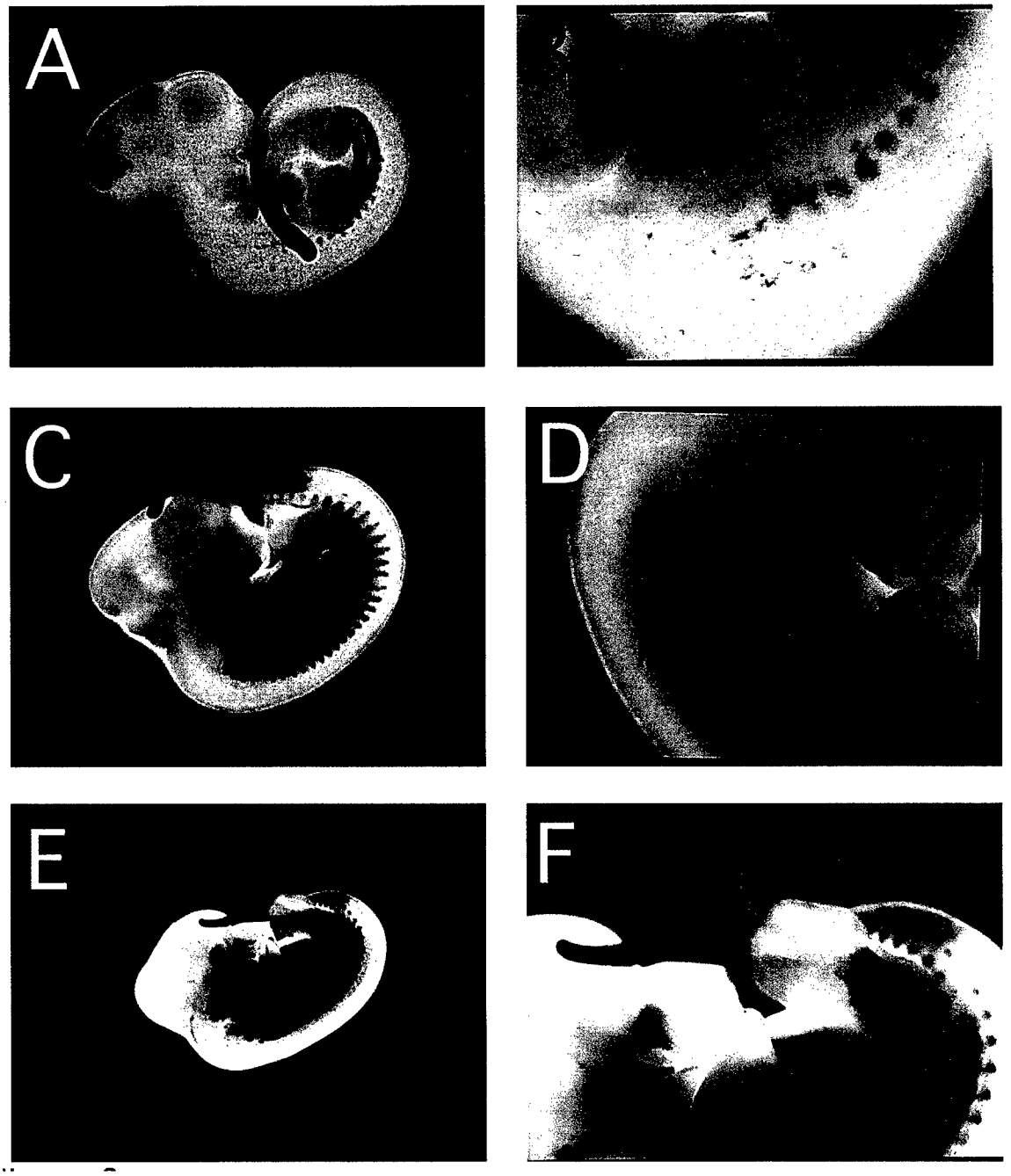

Figure 1. ß-gal staining of embryos carrying the 8 kb *Hoxb-13*/lacZ transgene. A, 10.5 days post coitum (dpc) embryo. B, magnified view of A; C, 11.5 dpc embryo; D, magnified view of C; E, 12.5 dpc embryo; F, magnified view of E.

Expanding the search for hoxb-13 cis acting control elements. The lack of prostate expression in transgenic mice carrying the 8 kb/lacZ construct prompted us to expand the search for cis-acting control elements by adding sequences both farther upstream as well as downstream of the *Hoxb-13* translational start site. The strategy we adopted was to insert the lacZ gene near the translational start site in a fragment that contained an additional 2.5 kb of upstream sequences and 2 kb of DNA downstream of the start site. At this juncture, our efforts to further characterize *Hoxb-13* cis acting elements were slowed by cloning difficulties.

The 12.5 kilobase EcoRI-BspHI fragment that was our target for the next lacZ transgene proved to be refractory to cloning in a series of conventional plasmid vectors and bacterial strains of various combinations. Vectors employed included pBluescript (Stratagene), pGEM-Teasy (Promega), and pZero-2 (Invitrogen). Bacterial strains included supercompetent DH10B, XL1 Blue, and XL-10Gold. These experiments failed in the hands of a research associate with more than 12 years of cloning experience, and other constructs with similar insert sizes were routinely generated during the same 12 month period.

Hypothesizing that the bacterial host could perhaps tolerate the *Hoxb-13* sequences in a lower dose, we attempted to clone the same fragment in a vector termed pACYC184. This plasmid, which has a p15A plasmid origin of replication, is a "medium" copy number plasmid that does not contain a polylinker region. Recombinants containing the *Hoxb-13* sequences that were "unclonable" in conventional vectors were obtained on the first attempt using pACYC184 as a vector. While these results allowed us to overcome the refractory-to-cloning impasse, we encountered new challenges in designing transgenes based on this vector. Due to the well documented negative effects of plasmid sequences on gene expression in transgenic mice, it is imperative to remove vector DNA from the fragment to be injected into embryos. The lack of a polylinker in pACYC184 made this critical requirement extremely difficult if not impossible.

Generation of a new transgene vector: To allow us to take advantage of the fact that pACYC184 was capable of supporting the replication of *Hoxb-13* sequences, we endeavored to modify the vector to incorporate a polylinker geared specifically to house mouse transgenes. To this end,

we designed a polylinker that contained 13 centrally located unique 6-base restriction sites, flanked by a series of 8-base restriction sites, two of which were duplicated to allow for one-step removal of vector sequences (Figure 2). The extreme 5' and 3' ends of the polylinker contain a 34-base recognition site for an intron-encoded endonuclease, which can also be used to release vector sequences if the 8-base sites occur within the transgene insert (Figure 2). This polylinker was generated using a PCR SOEing strategy, and was inserted into a derivative of pACYC184 that had been reduced in size by elimination of 2 kb of unnecessary sequences. The resulting polylinker was sequenced to confirm its authenticity and the lacZ gene was inserted into its center. This effort, though costly in terms of time, was absolutely required for us to move forward in the search for cis-acting sequences from the human *Hoxb-13* gene.

Activity of a 12.5-kb *Hoxb-13*/lacZ transgene. The pACYC184-based construct containing lacZ inserted at the translational start site within a 12.5 kb region of the *Hoxb-13* locus was injected into FVB embryos to derive transgenic mice. The impending date for submission of our phase II proposal precluded us from analyzing transgenic F1 mice carrying this construct, so we opted instead to euthanize founder transgenic mice to characterize gene expression during embryogenesis and in the prostate gland of postnatal mice. Analysis of one litter of 8 embryos arising from fertilized eggs injected with this construct showed one 10.5 dpc embryo with ß-gal activity in the anticipated *Hoxb-13* pattern (data not shown). Ectopic expression of the lacZ gene in myotomes was not observed in this embryo. This observation confirmed that the transgene was functional and capable of expressing the lacZ gene and that a negative regulatory element that suppresses ectopic expression in somites was present.

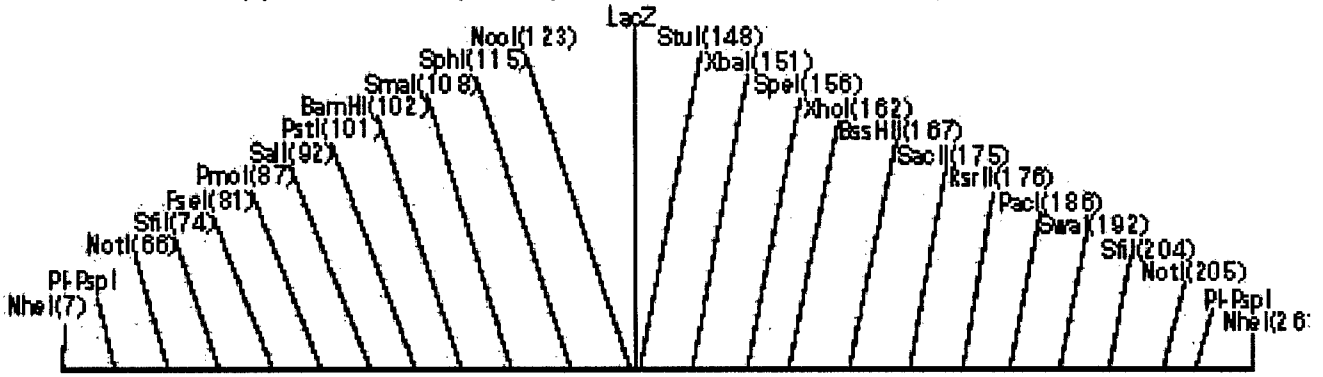

Figure 2. Polylinker region of the modified pACYC-184 vector, called pEGRA. The total length of the polylinker is 270 bp.

Of 64 founder generation animals born, 4 female and 4 male transgenics were identified by Southern blot analysis. Following prostate dissection, half of each gland was processed for analysis of ß-gal activity and RNA was extracted from the other half. Posterior colon (including rectum) and seminal vesicles were also harvested and processed similarly. Identical sets of tissue from non-transgenic littermates served as negative controls. Whole mount staining of tissues from transgenic and non-transgenic mice failed to reveal ß-gal activity in the transgenic prostate glands that was measurable above the background level of endogenous ß-gal activity (data not shown). In addition, Northern blot analyses of RNA extracted from transgenic tissues failed to detect lacZ mRNA.

Continuing the search for prostate regulatory elements-Genomic Analyses of the Hoxb-13 locus: Given the lack of transcriptional activity in the prostate of the reporter gene mice with 12.5 kb of *Hoxb-13* sequence, we attempted to delineate the intergenic region between *Hoxb-13* and its nearest upstream and downstream neighbors. The approach used was to use the Basic Local Alignment Search Tool (BLAST) to compare a sequence file containing 80 kb of sequence from the *Hoxb-13* locus against a human expressed sequence tag (EST) database. These analyses revealed three transcripts that mapped to within 21 kb of *Hoxb-13.* The closest was located 6 kb downstream of the *Hoxb-13* transcriptional start site. At this time, a report appeared in the literature describing PRAC, a novel human gene with an expression pattern that closely matched that of *Hoxb-13* (1). The other two cDNA clones were screened for expression in normal adult prostate by Northern Blot analysis. The downstream transcript (AI346833), located 21 kb away, did not show expression in the prostate (Fig 2A), leading us to delineate this as a downstream endpoint for future transgene constructs. A cDNA derived from a region located 8 kb upstream of Hoxb-3 (BF681289), yielded weak expression of a 500 bp transcript in the human prostate (Fig2B). These analyses led us to conclude that cis-regulatory elements directing gene expression in the prostate were likely to lie between *Hoxb-13* and its downstream neighbor PRAC.

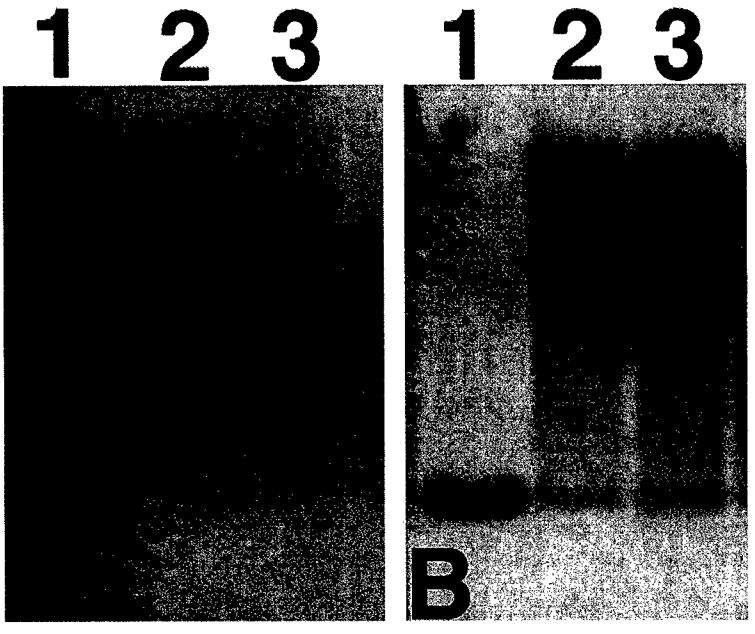

Figure 3. Northern Blot analysis of cDNA clone expression in prostate. Panel **A:** AI346833. Panel **B**: BF681289 . Lane 1, mouse ventral lobe; lane 2, whole human prostate sample#1; lane 3; whole human prostate sample #2

To attempt to capture cis-elements that mediate *Hoxb-13* and PRAC expression in the prostate, the 5 kb intergenic region downstream of *Hoxb-13* and upstream of the transcriptional start site of PRAC was amplified by PCR and cloned into the existing 12.5 kb construct to generate *Hoxb-13*/18.5kb. Correct insertion of the PCR product was confirmed by sequencing across the ligation junctions. Nine transgenic founder generation mice, including 4 males were generated. A panel of urogenital tissues from all of the transgenic males were stained for lacZ activity. To our great disappointment, none of the founder males carrying the *Hoxb-13*/18.5 kb construct showed lacZ activity in the prostate or any other urogenital organ. These data demonstrate that cis-acting elements capable of mediating gene expression in the prostate do not lie between *Hoxb-13* and its nearest downstream neighbor, PRAC.

Further analyses of the human Hoxb-13 transcription unit: While there is only one *hoxb-13* transcript reported for mice (2), previous experiments in our laboratory have demonstrated two transcripts for human *Hoxb-13* of 1.7 and 3.2 kb. The sequence of the 1.7 kb transcript has been

determined, but the structure of the larger transcript was unknown. To determine the nature of the 3.2 kb mRNA, upstream and downstream sequence flanking the reported 1.7 kb transcript were BLASTed against the dbEST to identify cDNAs from tissues known to express *Hoxb-13*. Seventeen EST clones were found that would correlate to a 3.2 kb transcript generated by differential polyadenylation. The largest of these clones (Genbank accession number AI700363) containing a cDNA insert distal to the polyadenylation signal for the 1.7 kb transcript was obtained,

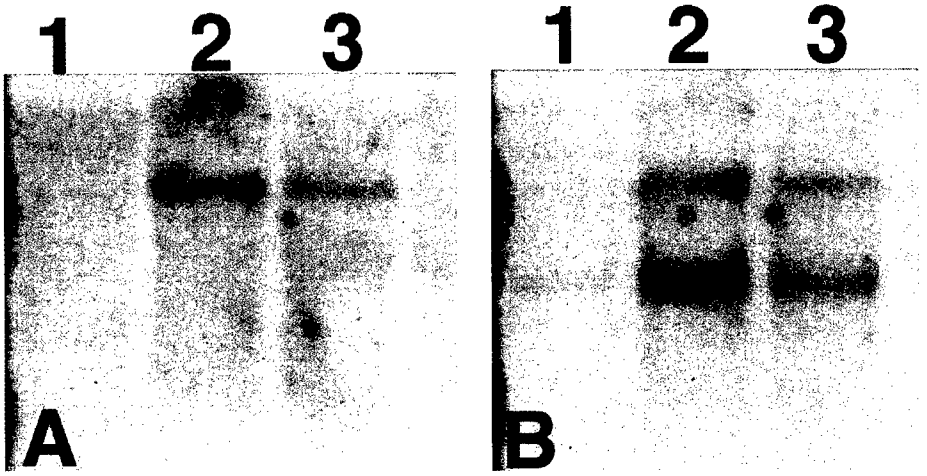

Figure 4. Panel **A:** AI700363 expression in normal adult prostate. Panel **B**: *Hoxb-13* expression in normal adult prostate; lane 1, mouse ventral lobe; lane 2, whole human prostate sample#1; lane 3; whole human prostate sample #2.

sequence verified, and used to probe a Northern blot containing RNA from normal human prostate (Fig. 4). A single hybridizing component of 3.2 kb was observed. The same blot was then stripped and hybridized with a probe from the proximal region of the 3' UTR. As expected, this probe hybridized to both a 3.2 kb band, and a 1.7 kb band.

Identification of a Hoxb-13 antisense transcript expressed in the prostate: We have recently obtained convincing evidence for the presence of an evolutionarily conserved antisense *Hoxb-13* transcript. As part of a collaboration with Dr. Gail Prins at the University of Illinois, Chicago, and under the auspices of another project, we had cloned exon 1 of the rat *Hoxb-13* gene. In the course of in situ hybridization experiments, Dr. Prins' lab observed that both antisense and sense *Hoxb-13* exon 1 synthetic RNA probes (Riboprobes) hybridized specifically to prostate

epithelial cells, suggesting that an antisense mRNA that includes at least part of exon 1 may be generated from the rat *Hoxb-13* gene. To confirm this observation in rat, and to determine whether an antisense transcript is present in mouse prostate, a single-stranded sense probe was generated by linear PCR from exon 1 of the mouse *hoxb-13* gene and used to probe a Northern blot containing rat and mouse prostate RNA. This probe readily detected a band of approximately 1.7 kb in both rat and mouse prostate RNA, indicating that an antisense mRNA was present (Figure 5). To determine whether a human antisense *Hoxb-13* transcript exists, the dbEST was searched for a corresponding antisense cDNA. Several human prostate ESTs were found that corresponded to an antisense human mRNA generated from the *Hoxb-13* locus. To determine the transcriptional start site of the human *Hoxb-13* antisense transcript, a 5' rapid amplification of cDNA ends (RACE) analysis was performed using RNA from LNCaP cells. Using a specific primer in exon 1 for reverse transcription, a 500 bp RACE product was observed (data not shown).

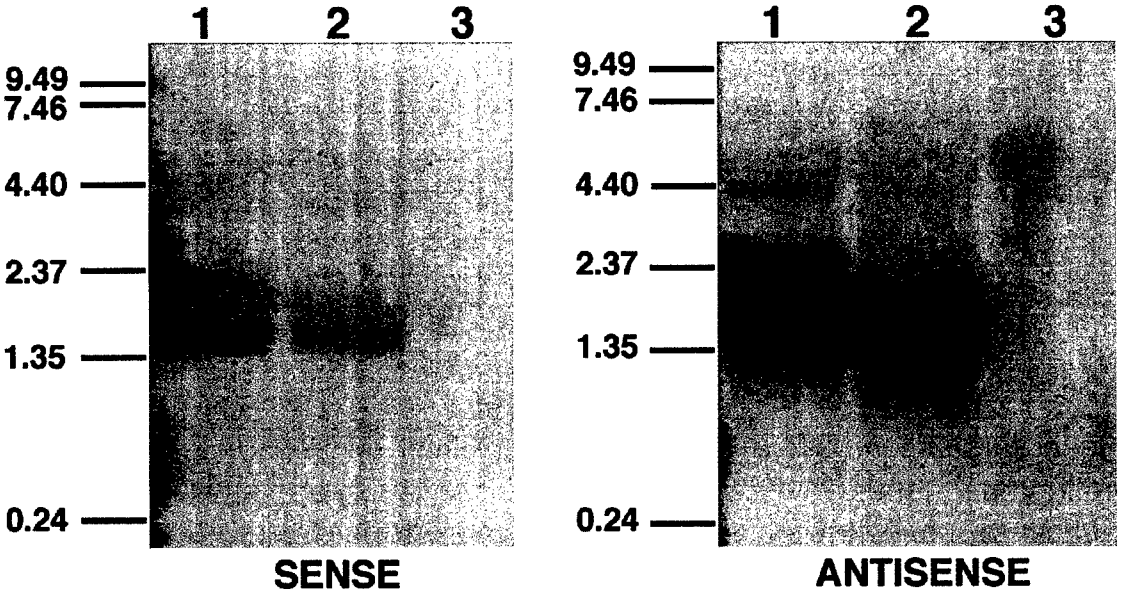

Figure 5. Northern blot analysis of rat and mouse prostate RNA. The left panel (sense) was hybridized with a single-stranded sense DNA probe revealing the presence of an antisense *Hoxb-13* mRNA. The right panel was hybridized with an antisense DNA probe.

This result suggests that the antisense start of transcription occurs within the intron of human *Hoxb-13*. The 5' RACE product has been cloned and is currently being sequenced. 3' RACE analyses will be used to determine the full cDNA sequence of antisense *Hoxb-13*.

Task 2

All of the experiments proposed in task 2 were predicated upon the successful completion of Task 1. The cloning and functional characterization 18.5 kb of sequence from the human *Hoxb-13* locus consumed our efforts but failed to reveal the presence of cis-regulatory elements capable of directing gene expression in the prostate.

KEY RESEARCH ACCOMPLISHMENTS

- mapping and sub cloning of human and mouse *Hoxb-13* genomic fragments
- construction of an 8 kb *Hoxb-13*/lacZ transgene
- generation of transgenic mice carrying the 8 kb *Hoxb-13*/lacZ transgene
- analysis of reporter gene expression in mice carrying 8 kb of *hoxb-13* sequences
- generation of a modified pACYC184 vector
- cloning of a 12.5 kb *hoxb-13* region that was refractory to cloning in conventional vectors
- construction of a 12.5 kb *hoxb-13*/lacZ transgene
- generation of transgenic mice carrying 12.5 kb *hoxb-13*/lacZ transgene
- analysis of reporter gene expression in mice carrying 12.5 kb *hoxb-13*/lacZ transgene
- analysis of the location of genes neighboring *Hoxb-13*
- characterization of the nature of the 3.2 kb and 1.7 kb *Hoxb-13* transcripts
- construction of a 18.5 kb *Hoxb-13*/lacZ transgene
- generation of mice carrying the 18.5 kb/lacZ transgene
- analysis of reporter gene expression in 18.5 kb/lacZ transgenic mice
- demonstration of the presence of an antisense *Hoxb-13* transcript in prostate

REPORTABLE OUTCOMES:
Manuscripts

Sreenath T, Orosz A, Fujita K, Bieberich CJ. (1999) Androgen-independent expression of *hoxb-13* in the mouse prostate. Prostate 41:203-7.

CONCLUSIONS: Although we have succeeded in identifying a promoter activity from the human *Hoxb-13* locus that directs expression in mouse embryos, we have been frustrated in our efforts to identify the cis-regulatory element(s) that are capable of directing expression in the prostate gland. Our survey of genomic sequences has covered 18.5 kb of the human genome, including 12.5 kb upstream, and 6 kb downstream of the *Hoxb-13* translational start site. Our efforts were temporarily slowed by the difficulties we encountered in cloning certain fragments of the *Hoxb-13* locus. However, we overcame that obstacle by constructing a vector with features that facilitate the cloning of "difficult" fragments. We suspect that we were not alone in having difficulty in cloning these regions in conventional vectors: we sequenced the ends of our pACYC184-based *Hoxb-13* clones and have BLASTed them against the deposited human genome sequence. Despite the fact that a "rough draft" of the human genome has been complete for several years, the *Hoxb-13* locus sequence has only recently been assembled in a contig and deposited in the database.

The fact that prostate cis-regulatory elements lie do not appear to lie within the 20 kb region around *Hoxb-13* strongly suggest that this gene is regulated in the prostate through the action of one or more locus control regions (LCRs). LCRs can be located at a considerable distance from the genes they regulate, on the order of 50-100 kb or more. The identification and functional characterization of LCRs for *Hoxb-13* will almost certainly require the use of much larger regions of the genome in reporter gene constructs in combination with biochemical approaches. For example, DNAse hypersensitivity mapping of a broad area around the locus may be required to reveal the location of potential LCRs. In addition, it is likely that bioinformatic approaches, which have only recently become feasible for *Hoxb-13*, will greatly facilitate the search for LCRs. The availability of a large sequence contig for human, and the assembly within the past month several months of a similar contig for the

mouse genome will greatly facilitate our future efforts to determine the location of prostate cis-regulatory elements that control *Hoxb-13*. Using web-based sequence alignment and analysis tools, we have already begun

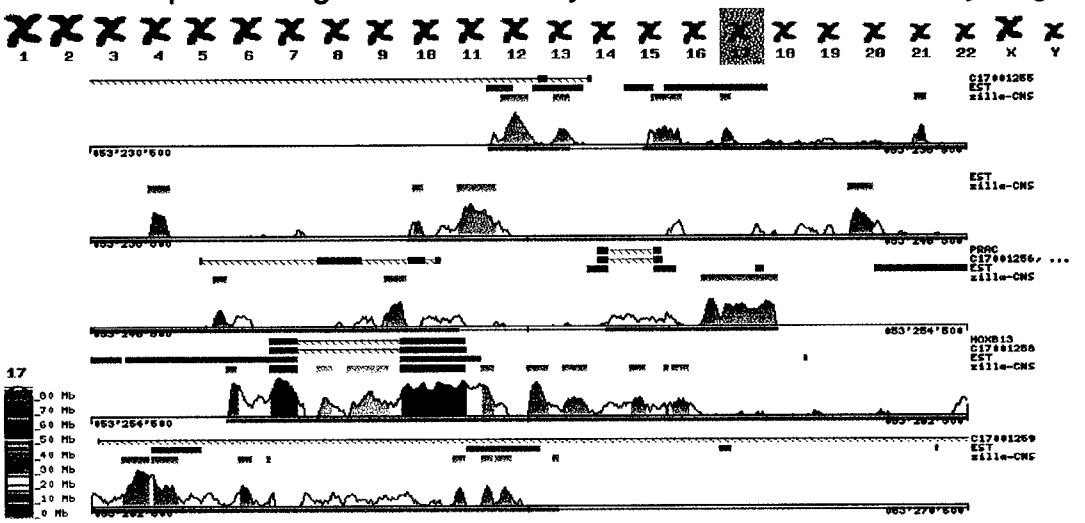

Figure 6. The Zbrowser graphical view of genome comparison (http://pipeline.lbl.gov) shows a comparison of human to mouse sequence containing *Hoxb-13*. The X-axis consists of 37.5 kb of sequence showing annotations of human genes and ESTs in blue and green. The Y-axis shows the percent homology above 50% of the corresponding mouse sequence to the human, calculated from 40-bp windows. Areas of yellow indicate 5' and 3' UTRs. Pink areas indicate conserved intronic sequence, while orange areas indicate noncoding regions that are phylogentically conserved.

to perform phylogenomic comparisons of mouse and human sequence to identify conserved features that might represent regulatory elements (Figure 6).

Our reporter gene analyses of a nearly 20 kb region of the human genome have pushed the limits of conventional cloning methods. It is difficult to envisage strategies that will allow us to assay larger regions using this approach. Future analyses will likely employ recombinational approaches to search for cis-regulatory activity in the context of larger regions of the genome (3). To this end, we have begun construction of recombinogenic vectors that will allow us to place the lacZ gene into a precise location in the *Hoxb-13* locus by homologous recombination in the context of a cosmid, PAC or BAC. This system uses a yeast/bacteria shuttle vector that we have recently used successfully to identify cis-regulatory elements that control the expression of another prostate-restricted transcription factor, NKX-3.1.

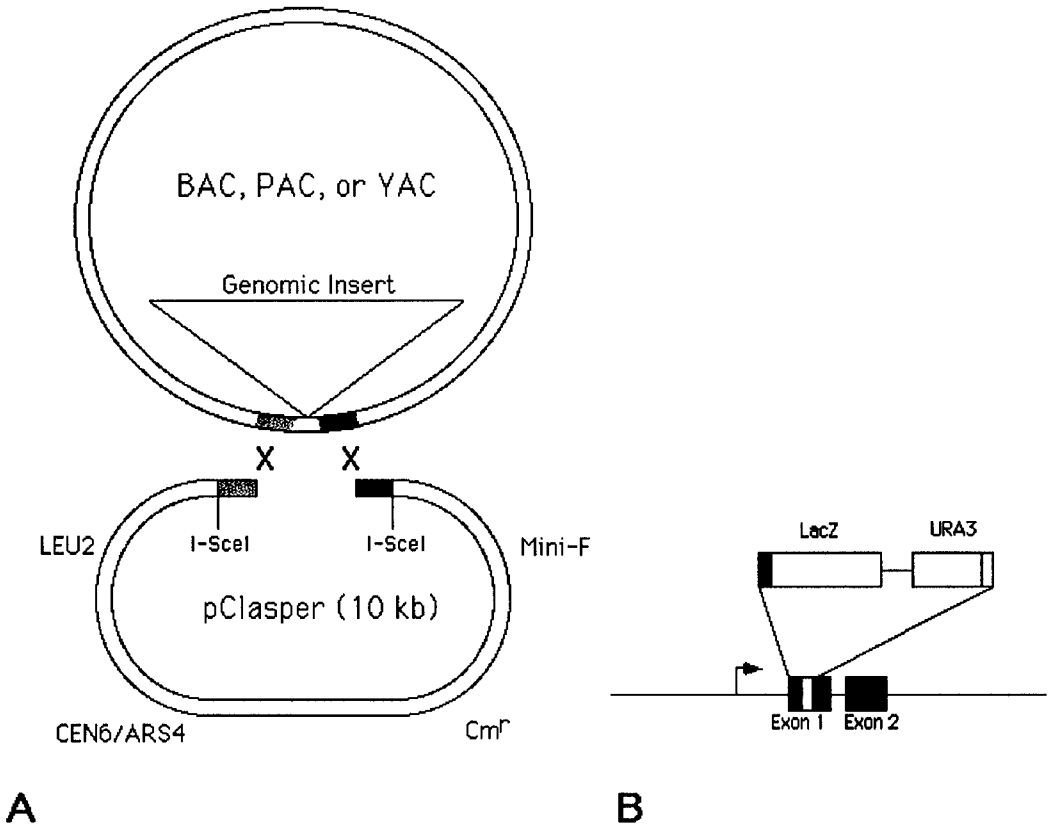

Figure 7. Panel **A**: Recombination of genomic insert into pClasper. Colored regions reflect homologous regions generated by PCR that flank sequence of interest. Panel **B**: Recombination of reporter gene/selectable marker into Exon 1 of a gene.

Several alternative hypotheses can be put forward to explain our inability to locate prostate cis-regulatory elements from the *Hoxb-13* gene. Although we consider it highly unlikely, it is formally possible that the human regulatory elements that confer transcriptional activity in the prostate do not function properly in mice. To test this hypothesis, we plan to search for regulatory elements from the mouse *hoxb-13* gene, then use phylogenomic comparisons to locate similar elements in the human locus.

A second hypothesis is that the presence of an antisense transcript for *Hoxb-13* could result in an antisense mRNA being generated from the lacZ reporter gene. In fact, this is likely to occur given that the antisense promoter appears to lie within the intron, and the lacZ gene is inserted

upstream near the ATG codon. If both sense and antisense mRNAs for lacZ are generated from the reporter gene, then the double stranded RNA for this relatively A/T rich bacterial gene could be rapidly degraded by the cell. We have not looked for the presence of an antisense *Hoxb-13* mRNA in embryos, where our reporter genes are clearly functional. It is possible that the antisense promoter is active only in prostate epithelial cells, and could represent a intriguing mechanism for regulating *Hoxb-13* expression in the prostate. To test this hypothesis ,we will generate transgenes that contain sequence from up and downstream of *Hoxb-13*, but which lack the intron.

Despite our lack of overt success in identifying prostate-specific, androgen independent regulatory elements for *Hoxb-13*, we have learned a great deal about the regulation of this gene and the structure of the RNAs is generates, including an antisense transcript. We remain fully committed to identifying the elements we set out to find at the outset of this work. Our efforts will be tremendously facilitated by the very recent public availability of a large genomic sequence for both mouse and human *Hoxb-13* loci. It is unfortunate that we did not have access to this information, which almost certainly existed in privately held databases for a considerable portion of the granting period, sooner.

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APPENDICES:

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The Prostate 41:203–207 (1999)

Rapid Communication

Androgen-Independent Expression of *hoxb-13* in the Mouse Prostate

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BACKGROUND. Hox genes encode transcriptional regulatory proteins that are largely responsible for establishing the body plan of all metazoan organisms. A subset of Hox genes is expressed during the period of organogenesis and into adulthood. *hoxb-13* is a recently-described member of the Hox gene family that is expressed in the spinal cord, hindgut, and urogenital sinus during embryogenesis.
METHODS. Northern blot and in situ hybridization analyses of *hoxb-13* expression in adult mouse tissues were performed.
RESULTS. *hoxb-13* mRNA is restricted to the prostate gland and distal colon in adult animals. In situ hybridization of mouse prostate tissue demonstrated that *hoxb-13* is expressed in the epithelial cells of the ventral, dorsal, lateral, and anterior prostate lobes. Accumulation of *hoxb-13* mRNA is not diminished following castration.
CONCLUSIONS. These data demonstrate that *hoxb-13* expression is androgen-independent in mouse prostate glands. The identification of *hoxb-13* as an androgen-independent gene expressed in adult mouse prostate epithelial cells provides a new potential target for developing therapeutics to treat advanced prostate cancer. *Prostate 41:203–207, 1999.*

KEY WORDS: **homeobox gene; gene expression; castration; cancer**

INTRODUCTION

The mammalian Hox genes are homologs of *Drosophila* homeotic genes that encode homeodomain transcription factors [1]. Gain and loss of function analyses have demonstrated that Hox genes in mammals play a critical role in establishing the basic body plan, for example, by patterning the axial and the appendicular skeleton and parts of the central and peripheral nervous systems during embryogenesis [1–3]. An intriguing feature of some Hox genes is their continued expression during organogenesis and in differentiated organs in adult animals [1]. Although their roles in pattern formation during early embryonic development have been studied extensively, their roles in later developmental events and tissue maintenance have received comparatively little attention. The importance of understanding their functions in differentiated cells is underscored by the recognition that deregulated expression of Hox and other classes of homeobox-containing genes have been implicated in oncogenic transformation of cultured cells and in tumors [4–9].

Several members of the Hox family of homeobox genes have been found to be expressed in human prostate cell lines [6] and in mouse embryos in the region of the urogenital sinus that gives rise to pros-

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tate glands [10,11]. *hoxd-13* has been shown to be expressed in the urogenital sinus during late gestation and in early postnatal mouse prostate glands, but is repressed at maturity [10]. A loss-of-function mutation of *hoxd-13* in mice results in a decrease in size and ductal branching of the dorsal and ventral prostate lobes [12]. Both *hoxc-11* and *hoxb-13* have been reported to be expressed in the prostatic region of the urogenital sinus at 12.5 days gestation, but their distribution at later stages and in adult tissues have not been described [11,13].

We have characterized the distribution of *hoxb-13* mRNA in adult mouse tissues and have found that it is expressed at a high level in the prostate gland and distal colon.

MATERIALS AND METHODS

RNA extractions and Northern blot analyses were performed essentially as described [14] using either a 4.5 kb probe containing the entire *hoxb-13* coding region (T.S. and C.J.B., unpublished) or a 550 bp *Apa*I-*Eco*RI restriction fragment containing only exon 1. Identical results were obtained with both probes. Dissection of prostate glands into component lobes was performed as described [15]. Orchiectomy was carried out on 6-week-old CD-1 mice as described for rats [16], and RNA was extracted from whole prostate glands pooled from two mice at each time point. In situ hybridization was performed as described [17] using 0.45 kb *Bgl*II-*Xba*I fragment derived from the 3′ end of *hoxb-13* [13].

RESULTS AND DISCUSSION

To determine whether *hoxb-13* is expressed in adult tissues, Northern blot analyses were performed. A 4.5 kb probe derived from the *hoxb-13* genomic locus and containing the entire transcription unit was hybridized to total RNA extracted from 18 different tissues of adult FVB mice (Fig. 1 and data not shown). A strongly hybridizing 3.2 kb component was observed only in RNA from the prostate and the distal region of the colon (Fig. 1A,B). Within the prostate gland, *hoxb-13* mRNA was detected in all lobes (Fig. 1A). When normalized to the level of a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the ventral and lateral lobes showed a similar steady-state level of expression, while in the coagulating gland (anterior prostate) and dorsal prostate the level was consistently 2-3 fold lower. A distal-to-proximal gradient of *hoxb-13* mRNA was observed in the colon (Fig. 1B). The rectum and 1 cm of adjacent colon showed the highest level of expression, while in the most proximal 1 cm of colon, just distal to the cecum,

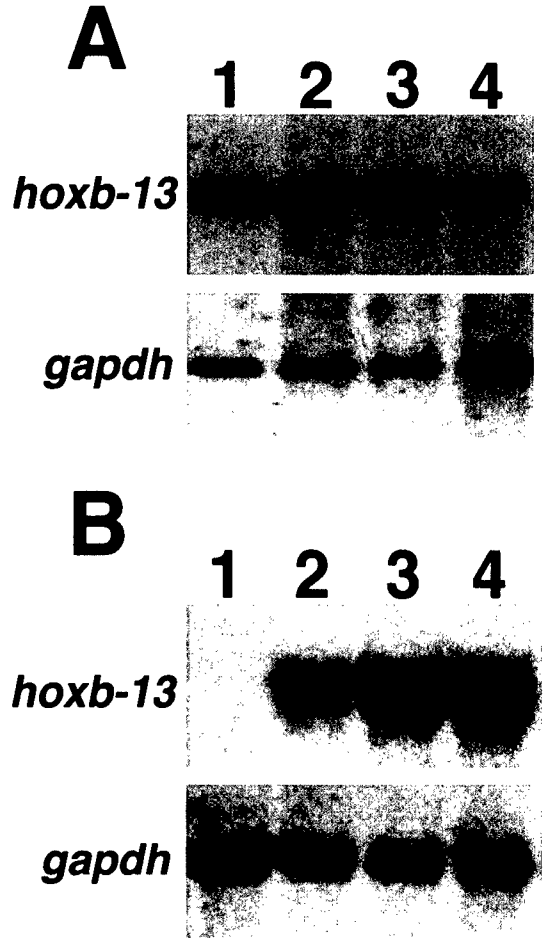

Fig. 1. Northern blot analysis of *hoxb-13*m RNA expression in adult mouse tissues. Panel **A**: lane 1, coagulating gland; lane 2, ventral prostate; lane 3, lateral prostate; lane 4, dorsal prostate. Panel **B**: lane 1, proximal colon, defined as 1 cm of intestinal tissue just distal to the cecum; lane 2, middle colon, defined as the central 1 cm of tissue between the distal margin of the cecum and the anus; lane 3, distal colon, defined as 1cm of colon adjacent to the rectum; lane 4, rectum. Average length of colon from the distal margin of the cecum to the anus was 8 cm.

hoxb-13 mRNA was not detected (Fig. 1B). *hoxb-13* mRNA was also detected in the central region of the colon at a level two-fold lower than in the rectum (Fig. 1B). Expression of *hoxb-13* was not detected by Northern blot analysis in other urogenital tissues including kidney, testis, urethra, bladder, seminal vesicle, ampullary gland, vas deferens, ovary, or uterus (data not shown). *hoxb-13* expression was also not detected in RNA from preputial gland, liver, spleen, lung, heart, thymus and brain (data not shown).

To characterize the cellular distribution of *hoxb-13* mRNA within the mouse prostate gland, in situ hybridization analyses were performed on histological sections of adult tissue. As predicted by the Northern blot analyses, hybridization to the *hoxb-13* probe was observed in all prostate lobes (Fig. 2 and data not

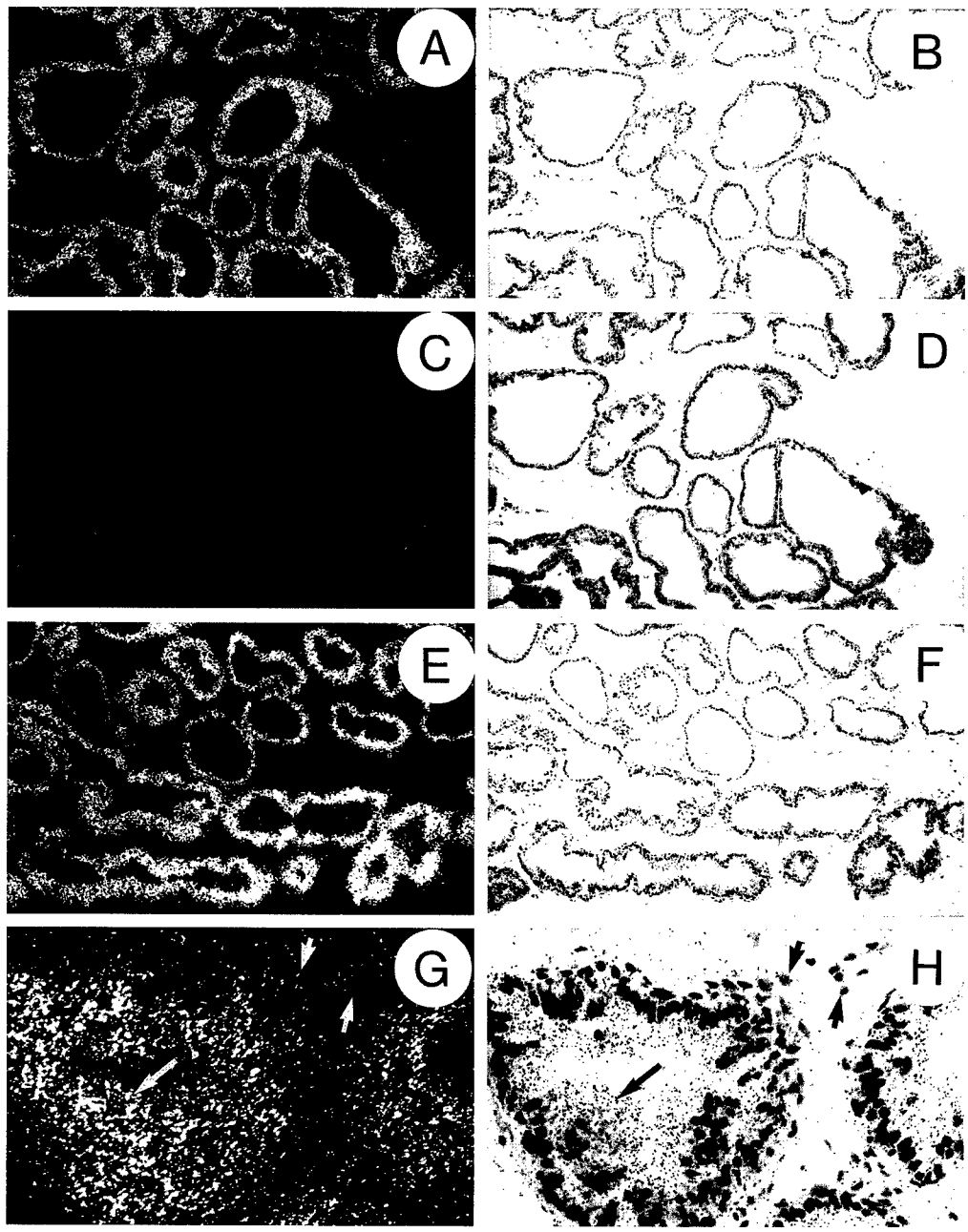

Fig. 2. In situ hybridization analysis of *hoxb-13* expression in adult prostate. Paraffin sections of ventral (A-D) and lateral (E-H) lobes. **A**, darkfield, **B**, brightfield low magnification view shows signal over ducts in the ventral lobe using the antisense probe. **C**, darkfield, **D**, brightfield view shows lack of signal with a sense probe on a serial section, demonstrating the specificity of the assay. **E**, darkfield, **F**, brightfield low magnification view of lateral lobe ducts. **G**, darkfield, **H**, brightfield high magnification view of a lateral duct shows hybridization signal over epithelial cells. Short arrows highlight stromal cells, long arrow points to epithelial cells.

shown). Hybridization signal was seen along the length of the ducts but was excluded from the proximal region of each main duct near the junction with the urethra. Within the ducts, strong hybridization signal was observed over luminal epithelial cells, while stromal cells did not display signal that was distinguishable from background.

The effect of castration-induced androgen deprivation on the steady-state level of *hoxb-13* mRNA was examined by Northern blot analysis. Surprisingly, the level of *hoxb-13* mRNA was not diminished within 8 days after orchiectomy when normalized to the level of GAPDH (Fig. 3 and data not shown). In contrast, expression of *mp12*, an androgen-dependent gene which encodes a protease inhibitor, decreased 70-fold 24 hours after castration and was not detectable after

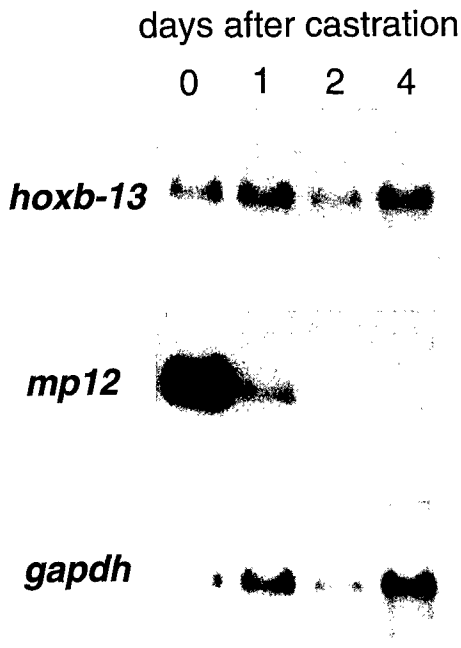

Fig. 3. Northern blot analysis of *hoxb-13* expression in orchiectomized mice. The same Northern blot membrane was sequentially hybridized with the indicated probes.

48 hours (Fig. 3) [18]. Similarly, the level of mRNA encoded by *nkx-3.1*, a homeobox gene that has been demonstrated to be androgen-dependent, was decreased nearly 10-fold within 24 hours, and 30-fold after 4 days (data not shown). These data indicate that maintenance of a high steady-state level of *hoxb-13* mRNA in the prostate gland does not require testicular androgens. It has been demonstrated in rats that a significant proportion (~20%) of ventral prostate epithelial cells survive at least 1 week after castration [19]. Our observations suggest that *hoxb-13* expression persists, and may even be up-regulated in the mouse prostatic epithelial cells that survive after castration.

Despite the fact that it is the most diseased organ in the human body, the genetic basis of prostate development, differentiation, and maintenance is not well understood. Considerable effort is currently focused on defining the role of androgens and peptide growth factors in prostate development and disease [20]. However, the nature of the genetic program(s) active in epithelial cells that underlie differentiation and maintenance remain largely undefined. Recently, several homeobox genes have been strongly implicated in both normal and malignant growth of prostate epithelial cells. The mouse *nkx-3.1* homeobox gene has been found to be expressed in developing and mature prostate glands where it is restricted to ductal epithelial cells and is androgen-dependent [14,21,22]. *nkx-3.1* is the earliest known marker of prostatic epithelial cells, and a loss-of-function mutation results in reduced ductal branching in all prostate lobes as well as defects in secretory protein production [23]. *nkx-3.1* null mice also develop epithelial hyperplasia and dysplasia in the anterior and dorsolateral lobes that increases in severity with age, suggesting that this homeobox gene may be a candidate tumor suppressor gene [23]. In contrast, the homeobox genes GBX1 and GBX2 have been found to be overexpressed in prostate cancer cell lines, and reduction of GBX 2 expression results in decreased clonogenic ability in vitro and tumorigenicity in vivo [6]. Together these data suggest that both gain and loss of function of certain homeobox genes may play a role in prostate cancer progression. Interestingly, human *hoxb-13* expressed sequence tags have been observed in both prostate and colon carcinomas [24].

Although most prostate tumors initially respond to androgen deprivation therapy, nearly all return as androgen-independent disease. Currently, there is a dearth of effective treatment options available to patients with advanced prostate cancer. The need to identify new potential targets for therapeutic intervention and to develop novel therapies to treat androgen-independent prostate cancer is axiomatic. Our observation of androgen-independent expression of *hoxb-13* in the mouse prostate provides a new model to study the molecular basis of androgen-independent gene expression in prostate epithelial cells.

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